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Germ cell line during the seasonal sexual rest of clams: finding niches of cells for gonad renewal

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Abstract

Reconstitution and renewal of tissues are key topics in developmental biology. In this brief work, we analyzed the wintry spent phase of the reproductive cycle in the Manila clam *Ruditapes philippinarum* (Bivalvia, Veneridae) in order to study the gonad rebuilding that in this species occurs at the beginning of the warmer months. We labelled VASA homolog protein—a germ cell marker—and compared the histological observations of the spent phase with those of the previously analyzed gametogenic phase. In *R. philippinarum*, during the reproductive season, most of the body mass is represented by sack-like structures (acini) full of developing gametes. In that period, VASA-stained cells are present at the basal pole of the gut epithelium, in the connective tissue, and around the acini. We here show that during the spent phase large portions of the intestine lack such cell type, except for some areas showing a few faintly VASA-stained cells. Cells with similar nuclear morphology are present among loosely organized cells of connective tissue, sometimes as single units, sometimes in small groups, rarely partially organized in primordial gonadic structures. These observations match the findings of RNA-targeting studies that during the spent phase identified the source of bivalve germ cells within the connective tissue in the form of quiescent units, and add new information on the possible maintenance of VASA-stained, multipotent cells among the batiprismatic cells of the intestine during the whole life span of these bivalves.

Keywords: VASA; DEAD-box RNA helicase; multipotent stem cells; gonad development; *Ruditapes philippinarum*

Introduction

Germ cells represent the carriers of the genetic information across generations [1]. The capability of undergoing meiosis identifies germ cells, marking their separation from other cell lineages of the body, those constituting the soma. The early separation of germ cells [2] occurs through a set of signals, RNAs and proteins present in the primordial germ cells (PGCs), the first cells of the germ line that arise during development. These molecular determinants are included into the germ plasm—a portion of the cytoplasm of the germ cells—inherited from the female gamete

(preformation), or derived by induction and *de novo* synthesis in the embryo (epigenesis) [3]. One of the principal factors characterizing the germ plasm is the protein VASA that functions as a local RNA unwinder, translational regulator, and piRNA pathway mediator [4, 5]. However, VASA is often expressed also in stem cells, not only in germ cells, suggesting that it has a much broader function in developmental regulation [5-7]. Together with VASA, other "germ line factors" have been found outside the germ line, and this has led to an ongoing rethinking about the germ line/soma paradigm [8]. In particular, data from lophotrocozoans and echinoderms uncovered that some stem cells, stably undifferentiated during the post-embryogenesis life span, express a germline multipotency program (GMP) [9]. GMP consists of a gene set previously associated with germ cells (e.g.: *vasa*, *nanos*, *piwi*) that maintains the cells that express it able to produce several cell types, including the germ line. Adopting Solana's [8] nomenclature we are referring to those cells as primordial stem cells (PriSCs).

Bivalves feature multiple reproductive modes, from the most frequent gonochorism to several forms of hermaphroditism [10]. Nevertheless, they experience a consistent annual reproductive cycle that is strongly influenced by environmental conditions [11]. It consists of: 1) a *spent phase* in which the gonad is reabsorbed; 2) a *reconstituting phase* in which the gonadic tissue begins to reorganize in a branched structure, the typical form of the gonad; 3) a *gametogenic phase* that consists in the multi-step differentiation of germ cells inside the acini—the functional units of bivalve gonads—leading to the ripening of the gonad; and 4) a *spawning phase* in which gametes are broadcasted into the aqueous environment. After this phase, before the beginning of a new spent phase, the gonad may undergo a "redevelopment" with release of other gametes in the same season [12]. The time of occurrence of the different phases can slightly vary from year to year—even in the same geographical region—depending on environmental conditions (e.g. water temperature, food resources).

The spent phase is the less investigated stage of the cycle, but recent studies have pinpointed its potential value for the understanding of gonad rebuilding mechanisms in clams [13, 14]. In this work, we analyzed such phase in the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850), that in Po River Delta (Italy) occurs during the winter [15]. We investigated the distribution of germ cells analyzing the expression of VASPH, the VASA homolog of *R. philippinarum* [16], and comparing the results with what observed during the gametogenic phase.

Materials and Methods

R. philippinarum adult specimens were collected in Sacca di Goro (Northern Adriatic Sea, close to the Delta of the Po River) during the spent phase (beginning of February 2016), and compared with specimens in the reconstituting phase (April) and in the gametogenic phase (July) (2014-2015). For histological sample preparation, the entire body was processed for hematoxylin and eosin staining following the method in Bettini et al. [17]. Tissue samples for immunohistochemistry were processed as described in Milani et al. [18] with the following modifications: the entire body was collected from animals in the spent phase and fixed for 3.5 hours, while, given their larger body dimensions, pieces of the body of about 0.5-0.8 cm^3 were sampled from specimens in the gametogenic phase (July 2015-2016) and fixed for 3 hours. Details on antisera production as well as specificity tests and experimental controls are provided in Milani et al. [18]. Imaging of hematoxylin-eosin staining was performed with Olympus BH-2 microscope and BEL BlackL. 5000 digital camera, and immunofluorescence imaging with confocal laser scanning microscope (Leica confocal SP2 microscope), and using Leica software. A total number of 100 sections from 10 specimens was observed.

Result and Discussion

During the reproductive season, the bivalve anatomy generally envisages a strict association between the intestine and the newly formed, seasonal gonad [19]. In this species, the reproductive tissue develops during the reproductive season as a non-discrete organ located inside the connective tissue near the intestinal loops at the base of the foot (Fig. 1) [12].

During *R. philippinarum* spent phase, the intestinal epithelium shows cells with a nuclear morphology comparable to that of VASA-stained cells in the gametogenic phase, for dimension (~ 5 μm), round shape, and highly condensed chromatin, and their localization close to the basal membrane of the intestinal batiprismatic epithelium (Fig. 2a,b, arrowhead; see also Fig. 1). When such cells are recognizable in the spent phase, either the cells do not show any staining (Fig. 2a) or, in some limited areas of the intestine and of the connective tissue, they show a faint VASA labelling (Fig. 2c,d), compared with the strong labelling of the gametogenic phase (Fig. 2a-d). The staining is concentrated in the cytoplasm at one side of the nucleus (Fig. 2c), as observed in similar cells during the gametogenic phase (Fig. 2b,d), but the weak signal probably indicates that VASA expression is just starting. Single or grouped VASA-labelled cells present in the connective tissue of *R. philippinarum* have a similar morphology to the VASA-stained cells in the intestinal

epithelium, with the VASA immunolabelling concentrated in the cytoplasm at one side of the nucleus (Fig. 2c and insets). Also, characteristic formations inside the connective tissue, involving VASA-labelled cells arranged around empty cavities are visible in spent phase samples (Fig. 2e). It is reasonable that these structures represent the organization of gonadic primordia in which VASA-labelled cells proliferate and rebuild the gonad (Fig. 2f). The localization of VASA-labelled cells in the connective tissue is in agreement with what described by several authors who studied *vasa* transcripts using *in situ* hybridization in *Crassostrea gigas*, *Mytilus galloprovincialis*, and *Pinctada fucata* [20-22]. In particular, they called germ line stem cells (GSCs) the few cells that remain quiescent in the connective tissue during the non-reproductive season. In *R. philippinarum*, the report of VASA-stained cells in the connective tissue—GSCs, considering the nomenclature of the above-mentioned Authors—is accompanied by the observation of cells with similar morphology and VASA-staining localization within the intestinal wall. It is a matter of conjecture that these two populations of cells belong to the same lineage, and that they both participate to gonad reconstitution.

Recently it was hypothesized that during the gonad reconstituting phase, VASA-stained primordial stem cells (PriSCs) coming from the intestine may contribute to the rebuilding of reproductive tissue [14]. Actually, we suppose that the digestive epithelium could be a reservoir of multipotent cells—PriSCs—that can be deputed to multiple cell fate [14]. VASA-stained cells increase in number during gonad rebuilding and are present both in the intestine epithelium and in the connective tissue [13, 14]. On the one hand, it is easy to assume that the VASA-positive cells in the connective tissue—were the new gonadic tissue is usually formed—are source of new gametogenic cells (see Online Resource 1 for confocal images of the reconstituting phase). On the other hand, the massive proliferation of VASA-stained cells in the intestinal epithelium during the reproductive season is difficult to explain by gut renovation alone. Indeed, cells deputed to renewal and turnover of the gut wall are present in the intestinal epithelium [23]. However, given the presence in several locations (in the intestinal epithelium, near the basal lamina, in the connective tissue, and around acini) of cells with similar nuclear morphology and VASA staining, it can also be hypothesized that those cells in the intestine are multipotent cells. This is in some measure supported by recent studies on many animal species that describe VASA-positive cells as, more generally, primordial stem cells able to give rise to germ and somatic cell lines [9]. This hypothesis does not rule out the first one, in fact, being PriSCs capable of originating both somatic and germ cells, they could act in the maintenance of the intestinal epithelium [9, 23] and also contribute to the gonad rebuilding. The two processes are indeed linked, because they are driven by the same environmental factors: the abundance of food triggers gametogenesis, thus beginning the

reproductive season and the consequent increase in the need for massive nutrient assimilation. Moreover, in the spent phase, the proliferation of VASA-stained cells seems to be absent, given that their number is consistently extremely limited. This is in line with the reduced body mass of bivalves during this phase, size that increases with gonad rebuilding during the reproductive season [11]. In this context, the tight association between intestine and gonad can also facilitate nutrient exchange in support of germ cell proliferation [24].

Conclusions

The evidence obtained with VASA localization inspired us to propose a hypothesis that would be interesting to test in the future. We can advance a scenario about the localization of germ cell precursors in *R. philippinarum* (see Fig. 3 for a visual summary): at the beginning of the reproductive season, quiescent PriSCs within the intestinal wall begin to proliferate massively, and migrate into the connective tissue, contributing to the organization of the developing gonad. At the end of the season, the gonadic tissue undergoes reabsorption. VASA-expressing cells persist in the intestinal niche, while the cells already migrated into the connective tissue (GSCs) remain there, some surrounding empty cavities. According to our hypothesis, at the beginning of the following reproductive season, an increased expression of VASA and other germ line determinants triggers gonad development with the contribution of both the niches.

The investigation of cyclic events of development, like that here described, can provide some important inputs to the topic of reconstitution and renewal of tissues, especially in non-model organisms.

Compliance with ethical standards

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, in accordance with the Directive 2010/63/EU.

Conflict of interest

The authors declare that they have no competing interests.

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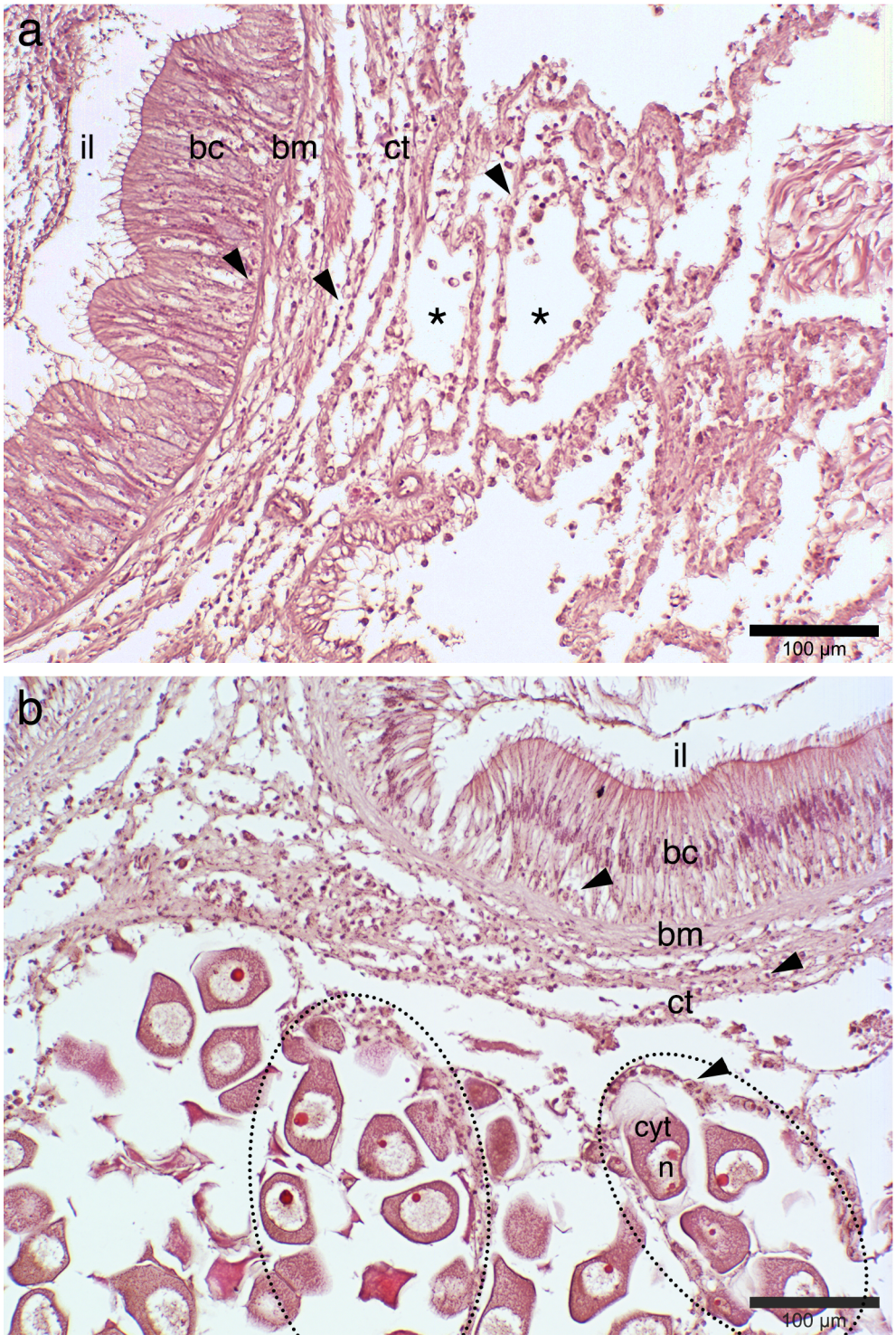
Figure legend

Fig. 1 *Ruditapes philippinarum* tissues stained with hematoxylin-eosin. (a) During the spent phase, a few cells with round nucleus (arrowheads) are present at the basal side of batiprismatic cells (bc) of the intestinal epithelium, near the basal membrane (bm), and some are also scattered in the connective tissue (ct) (il = intestinal lumen). Inside the ct, some empty cavities (*) surrounded by these cells are present. (b) During the gametogenic phase, the gonadic tissue is located inside the ct, near the gut, and consists of acinus-like structures (two acini are circled in dotted line) containing gametes (in this case oocytes at different stages of differentiation; n = nucleus, cyt = cytoplasm of one oocyte). At the basal side of bc, near the bm, small cells with a round nucleus are present; cells with similar morphology are also dispersed in the ct and at the acinus periphery (arrowheads).

Fig. 2 Immunolocalization of VASA in *Ruditapes philippinarum*
(a, c, e) Spent phase. (b, d, f) Gametogenic phase. (a) Intestinal wall lacking VASA-labelled cells, but showing cells with a round nucleus that in the gametogenic phase are VASA-stained (arrowhead). From top to bottom: intestinal lumen (il), batiprismatic cells (bc), basal membrane (bm), and connective tissue (ct). (b) Strongly VASA-labelled cells are present in the intestinal wall and in the ct (arrowheads). (c) Faintly labelled cells are present in the intestinal wall and in the ct (arrowheads). Inset (top left): magnification of a VASA-labelled cell close to bc. Inset (bottom right): magnification of a VASA-labelled cell within the ct. (d) ct with VASA-labelled cells (arrowhead). (e) Putative primordial acinus, with VASA-stained cells (arrowhead) defining its area, localized in the ct (transversal section). (f) Portion of a female acinus filled with oocytes (each circled in dashed lines; n = nucleus, cyt = cytoplasm of one oocyte), and VASA-labelled cells in the surrounding ct. Some of the VASA-labelled cells, those closer to oocytes, show an enlarged, stained cytoplasmic portion at one side of the nucleus (arrows). Red: VASA staining; green: nuclear staining. All the images are average projections.

Fig. 3 Visual abstract of *R. philippinarum* gonad rebuilding hypothesis. 1) During the spent phase VASA-stained cells are present in the intestinal epithelium (PriSCs) and in the connective tissue (GSCs). 2) When the reproductive season begins, the gonad develops inside the connective tissue by contribution of VASA-stained cells in the intestine that proliferate/migrate and by proliferation of GSCs in the connective tissue where gametogenesis starts. 3) Newly formed acini are progressively filled with differentiated gametes. The proliferating activity is reduced approaching the following spent phase, in which few VASA-stained cells remain localized in the connective tissue and in the intestinal epithelium.

Online Resource 1 Immunolocalization of VASA in *Ruditapes philippinarum* during the gonad reconstituting phase.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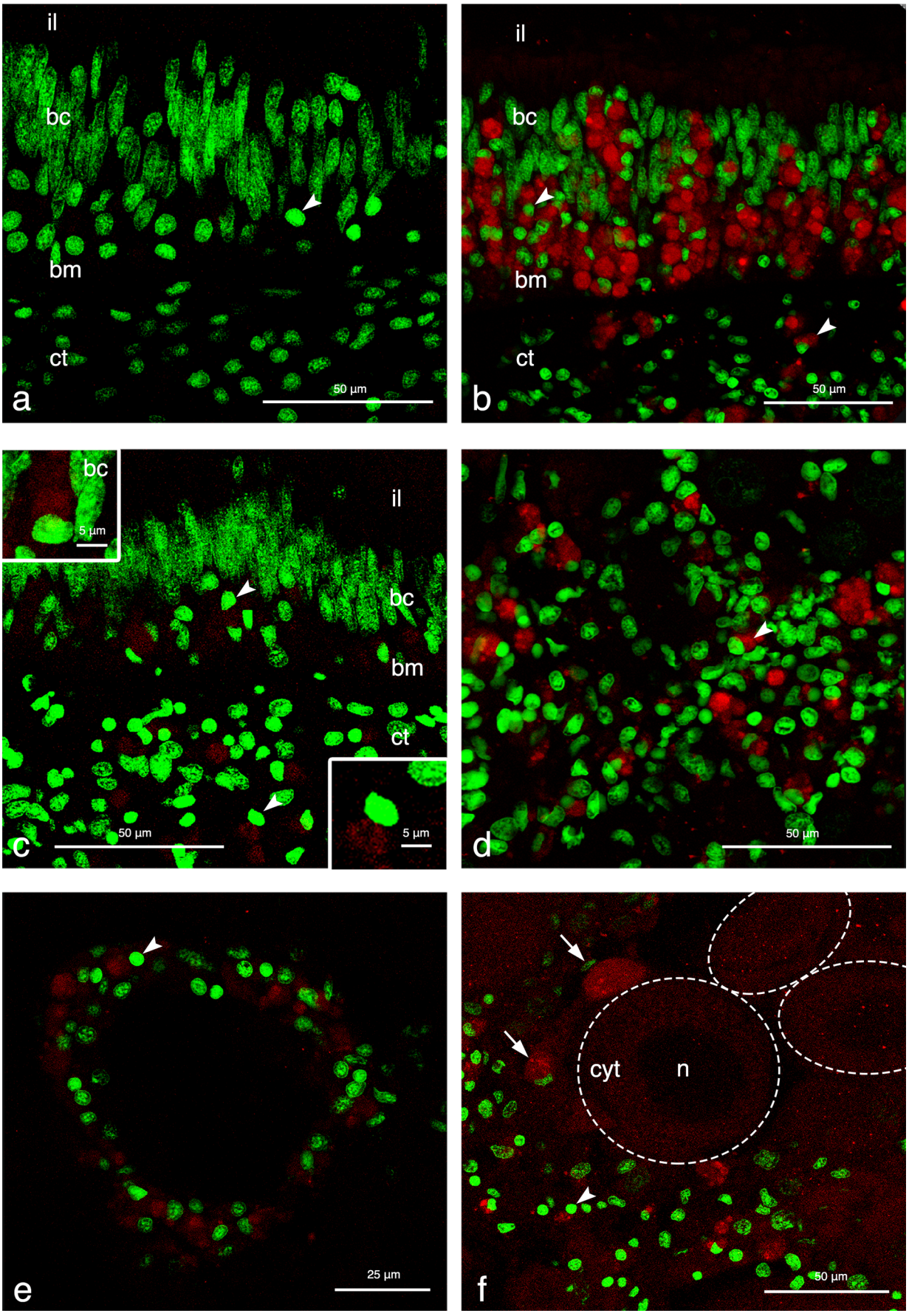
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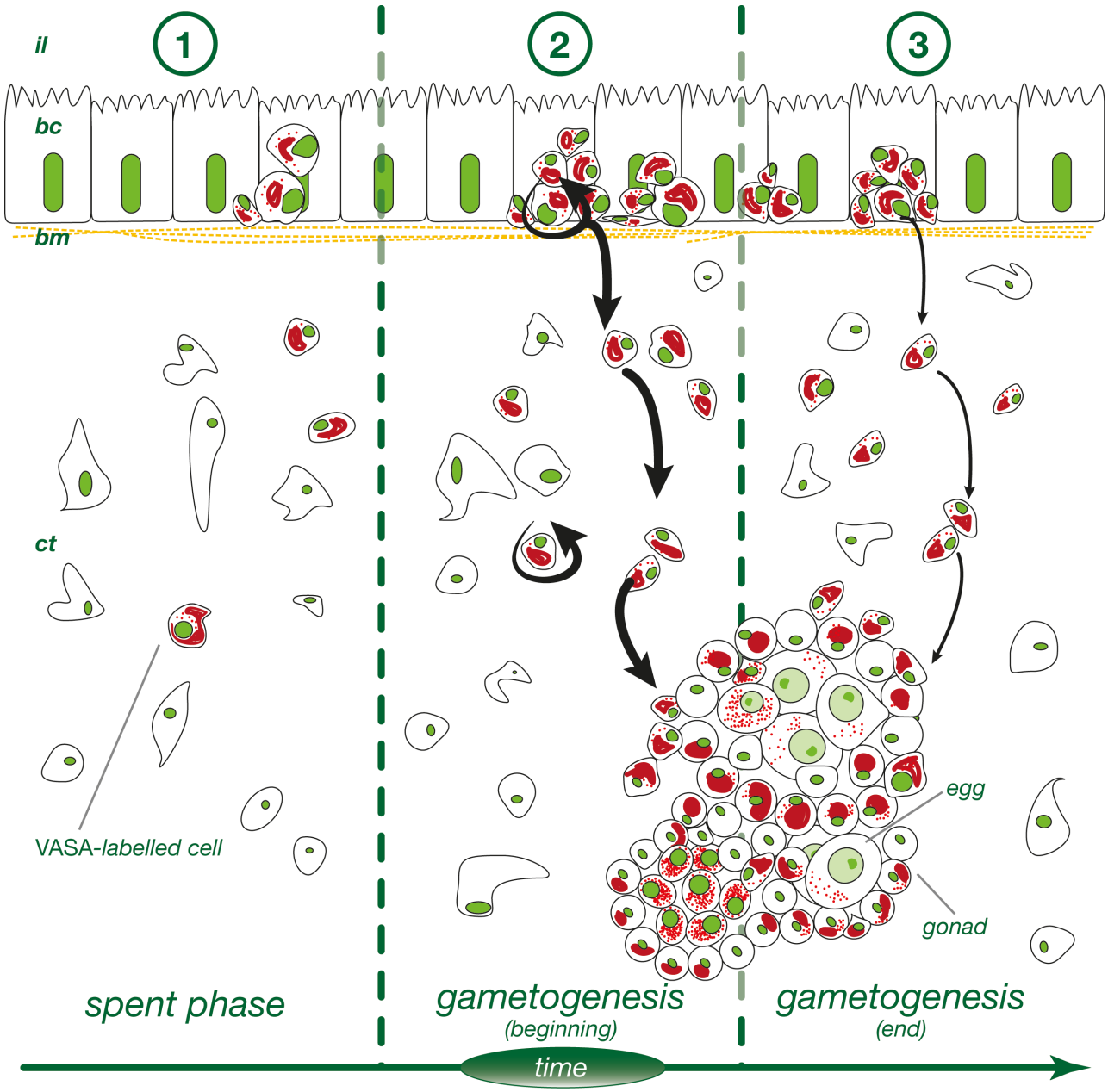
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